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OSMOTIC CONSTITUENTS OF THE BLOOD PLASMA AND PARIETAL MUSCLE OF *SQUALUS ACANTHIAS* L.

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The blood plasma of elasmobranchs is isosmotic or slightly hyperosmotic to sea water, but ions account for only part of its osmotic concentration, a large fraction being made up by urea and trimethylamine oxide (Holmes and Donaldson, 1969). These nitrogenous compounds are also present in high concentration in muscle (Smith, 1929; Dyer, 1952).

In this paper an attempt is made to outline the sea water-plasma and plasma-muscle steady states by comprehensive analyses of muscle and plasma of specimens of the spiny dogfish *Squalus acanthias*. Measurements of osmotic concentration of plasma and muscle have been made and compared with the sum of analyzed constituents, values of the latter, first obtained as milligram-ion or millimolar concentrations per kilogram solvent water, being converted to milliosmoles by the appropriate osmotic coefficients. Imprecision arises here owing to lack of knowledge of some coefficients, and because of the possibility that some of the constituents may be bound to protein, exerting little osmotic effect. Some idea of the amount of ion-binding in muscle has been obtained by analyses of the juice expressed from muscle by a tissue press or obtained by ultracentrifugation. Estimates have also been made of the extracellular space in muscle, thus enabling intracellular concentrations to be calculated.

Materials and Methods

Specimens of *Squalus acanthias* were caught by trawl in the Firth of Clyde and were kept in tanks of flowing sea water. Salinity during several summer periods varied from 32.2–33.4‰ (18.20–18.88 g Cl/liter) with temperatures of 9–12° C. After stunning the fish, blood was withdrawn by syringe or pipette under liquid paraffin from the heart and placed in centrifuge tubes kept in a beaker of crushed ice. After centrifugation, the plasma, still under paraffin, was removed and used for analysis. Samples of white parietal muscle were taken from the dorsal and lateral region of the tail just behind the second dorsal fin (epaxial muscles). After light blotting with filter paper, separate samples were used for cations (ashing at 550° C in the presence of sulphuric acid), determination of dry weight and the preparation of trichloroacetic extracts, tungstic acid extracts and zinc hydroxide extracts.

Methods for the analysis of Na, K, Ca, Cl and SO_4 of muscle and plasma were essentially those of Robertson (1949, 1960) with appropriate modifications for the lower concentration of ions in elasmobranchs. Magnesium was estimated by Heagy's (1948) method. Some of the analyses for Ca were done by atomic absorption spectrophotometry; interference by phosphate in the Ca estimation of muscle

and muscle juice was suppressed by lanthanum chloride at a final concentration in the solution investigated of 1% La ions. Lactate of plasma and muscle was determined in trichloroacetic acid filtrates (Hullin and Noble, 1953).

Ice-cold trichloroacetic acid muscle filtrates were used immediately for phosphate fractionation (Umbreit, Burris and Stauffer, 1949), the phosphorus of the fractions being determined according to Sumner (1944), and for determination of free and bound creatine (Ennor, 1957). TCA filtrates were also used for analysis of betaine and trimethylamine oxide (Kermack, Lees and Wood, 1955). For betaine gravimetric estimation of the mixed reineckates of those two compounds was substituted for colorimetry.

This was done in porosity 4 filter crucibles closed with rubber bungs. McIlvaine's buffer solution of pH 2.2 was saturated with ammonium reineckate, and further saturated with the salts of the two compounds by adding a little betaine-HCl and TMAO-HCl in solution. Twenty ml of the filtered solution were used, to which was added 1–2 ml of the muscle or plasma filtrate. After mechanical stirring for 5–6 minutes, the crucibles were left covered for 60 minutes at room temperature. After filtration the precipitate was washed thoroughly with n-propanol saturated with betaine and TMAO reineckates; in the first washing the precipitate was suspended and stirred with about 15 ml of the washing solution, the crucible being closed. Finally, the precipitate was dried with ether and the crucible weighed after 20 minutes in a desiccator. From the previously determined TMAO content of the filtrate and the molecular weight of TMAO-reineckate, 393.49, the amount of this compound in the precipitate is found. Any excess of precipitate is taken to be betaine reineckate, molecular weight 435.53, from which betaine is calculated.

In the TMAO analysis formaldehyde was added to hold back ammonia during the micro-diffusion of the trimethylamine. Tungstic acid filtrates were used in the estimation of the α-amino N of the free amino acids of muscle and plasma (Frame, Russell and Wilhelmi, 1943; Russell, 1945), and of creatinine of muscle and plasma (Owen, Iggo, Scandrett and Stewart, 1954).

Micro-diffusion techniques (Conway, 1962) were used in estimating bicarbonate and ammonium ions, urea (after urease) and total nonprotein N (after micro-Kjeldahl). Tungstic acid filtrates were used for muscle and for the NPN of plasma, but the two ions and urea of plasma were analyzed directly. Zinc hydroxide filtrates were used in the determination of glucose (glucose-oxidase method with Boehringer's Biochemica Test reagents) and of glycerol (Lambert and Neish, 1950).

Water contents were obtained by measuring the difference between fresh and dry weights, plasma samples being dried for 4 hr and muscle for 24 hr at 100–101° C.

A few measurements of extracellular space in muscle were obtained by injecting a solution of inulin in sea water intraperitoneally and taking samples of blood and muscle 14–22 hr afterwards. Inulin was estimated by measuring the fructose obtained after hydrolysis (Roe, Epstein and Goldstein, 1949).

The Krogh-Baldes pressure method (Krogh, 1939, p. 211) was used to measure total concentration (milliosmoles) of plasma and muscle juice, the juice being obtained with a small tissue-press (Krogh, 1938). Most measurements of the juice were made within 1.25 hr of removing the muscle, thus minimizing changes

due to breakdown of labile compounds. The osmotic concentration of the plasma and sea water was determined in specimens which had been equilibrated for 24 hr in water of constant chloride content.

Some analyses of ion were made on the muscle juice, and on fluid centrifuged from muscle. Centrifuging was done at 40,000 g (18,000 rpm) for 30–40 minutes at 0° C in polypropylene tubes. From 20 g muscle 5–6 g of centrifuged fluid of water content 88–89% could be obtained, leaving the residual muscle with about 68–70% water.

The following osmotic equivalence data are used (Robinson, 1954; Robinson and Stokes, 1965). A sea water of chlorinity 18‰, and salinity 32.52‰ is equivalent to 0.5263 molal NaCl with a concentration of 970 milliosmoles. At 20° C this sea water has a chloride concentration of 18.41 g Cl/liter.

Much of this work was done at the Millport Marine Station of the Scottish Marine Biological Association, before the move of the laboratory to its new site at Dunstaffnage, Oban.

Results

Osmotic concentration of blood and muscle

Seven measurements of the osmotic concentration of plasma and of the juice expressed or centrifuged from muscle were made in *Squalus* (Table I). The plasma was hyperosmotic to sea water by 0.3–5.2% and in six out of the seven,

Table I

Osmotic concentration of blood and muscle of Squalus
(*milliosmoles per kg water*).

No. and sex	Sea water	Blood plasma	Muscle juice	Plasma as % sea water	Muscle juice as % plasma
♂					
1	964	1005	997 (0.5 hr)	+104.3	99.2
2	968	989	1019 (0.5 hr)	+102.2	103.0
3	970	985	1006 (0.5 hr)	+101.5	102.1
4	968	971	1018 (1.2 hr)	+100.3	104.8
5	964	997	1013 (1.25 hr)	+103.4	101.6
6	958	1008	1087 (2.5 hr)	+105.2	107.8
Mean	965	993*	1023†	102.8	103.1
S.E.	±1.8	±5.6	±13.2	±0.75	±1.2
♀					
1	979	991	1030 (1.5 hr)	101.2	103.9
2	983	1004	—	102.1	—
3	983	1026	—	104.4	—
Mean	982	1007	—	102.6	—
S.E.	±1.4	±10.1	—	±0.95	—

Figures in parentheses are times between removal of muscle and completion of estimation. Muscle juice was obtained by tissue press from the 6 ♂♂, by means of the ultracentrifuge in the ♀.

* Significantly different from sea water by *t*-test ($P < 0.01$).

† Not significantly different from plasma ($P > 0.05$).

the muscle juice was hyperosmotic to the plasma. While the first difference is statistically significant (for the 6 ♂ specimens) the second is not, owing to the considerable variation. There is a tendency for the muscle juice to be more hyperosmotic the longer the completed time for preparation and estimation. Thus the 2.5 hr value of 107.8% that of the plasma is outside the mean + 2 S.D. of the remaining 5 values (106.2%) done within 1.25 hr. The higher values in the muscle juice are most probably due to some breakdown of labile constituents such as creatine phosphate.

Inorganic ions

Composition of plasma and whole muscle. Three comparisons of plasma, muscle and sea water showed fairly broad agreement, and the means are given in Table II. The sum of ions investigated in the plasma came to 51–59% of that in

TABLE II

Ionic composition of plasma and whole muscle in 3 ♂ specimens.

	mg-ions per kg water									Water (g per l or g per kg)
	Na	K	Ca	Mg	Cl	SO_4	Lactate	Total P*	Total	
Plasma	296	7.2	2.95	3.48	276	3.11	7.2	2.37	595†	959
S.D.	24.4	1.8	0.30	1.34	21.6	—	—	—	41	7
Muscle	42.4	119	2.09	12.9	35.9	1.24	23.8	91.3	329	761
S.D.	9.7	14.6	0.45	1.57	5.7	0.35	13.4	14.9	28	23
Sea water	453	9.6	9.9	51.6	529	27.3	—	—	1080	989

* mg-atoms.

† This total is slightly different from addition of mean plasma values (598), owing to absence of figures for SO_4, lactate and total P for one specimen.

sea water. The six major ions of the plasma are lower than the corresponding ions in sea water, the mean magnesium and sulphate levels being 7–11% of those in the surrounding water. Sodium, chloride and potassium levels are relatively much higher at 52–75%, while calcium is only 30%.

In muscle the total concentration of ions is rather more than half that of the plasma. The increased potassium and magnesium of muscle, about 17 and 4 times plasma values, are offset by lower concentrations of sodium and chloride, which are about a seventh and an eighth of those in the plasma. The acid-soluble phosphorus of muscle, with a mean of 91 mg-atoms, is present as various phosphate compounds which act as anions; these are considered further below (see Table VIII). No special precautions were taken in the estimation of lactate, so that the values may be higher than *in vivo*. A lower plasma concentration of 2.9 mg-ions was found in a female specimen.

Apparent extracellular spaces in muscle and intracellular ionic concentrations. Intracellular concentrations of ions in muscle could be calculated if the extracellular space were known. The technique of incubating strips of muscle in a

TABLE III

Apparent extracellular spaces in muscle (concentrations as percentages of those in plasma, on water content basis) and calculated intracellular chloride (based on inulin injection experiments).

Specimen	Extracellular spaces as per cent total muscle water		Chloride mg-ions per kg water			Time allowed for inulin equilibration hr
	Inulin	Chloride	Plasma	Whole muscle	Muscle cells	
♂	13.22	13.84	309	42.8	2.3	15
♂	18.18	24.33	345	83.8	25.8	19
♂	4.31	13.90	283	39.4	28.4	22
♀	14.75	22.42	271	60.7	24.3	14
Mean	12.62	18.62	302	56.7	20.2	—
S.E.	±2.95	±2.77	±16.4	±10.2	±6.0	

Ringer solution containing inulin as used for determination of extracellular space in amphibian and rat muscle (Ling and Kromash, 1966) proved valueless for dogfish muscle, even with the animal's own plasma as medium. Chloride values in the muscle rose to 2–3 times their initial values after 6–11 hr at 10° C, although the inulin spaces remained at about a third of the chloride spaces.

Some estimates were obtained by injecting a solution of inulin in sea water into 4 animals, and measuring the inulin and chloride concentrations of plasma and muscle after 14–22 hr (Table III). The mean inulin space was about two-thirds that of the chloride space, indicating that some chloride is present inside cells, but the calculated intracellular values are rather variable, 2.3–28.4, with a mean of 20.2 mg-ions/kg cell water. Most of the whole muscle and plasma chloride concentrations of these specimens are higher than those in Table II, the mean muscle chloride space of the latter being 13.02%, compared with 18.62%. If the inulin space as measured was used to calculate the intracellular ionic concentrations of the specimens in Table II, there would be no chloride inside the cells. To get an approximate idea of average intracellular concentrations in these specimens it would seem appropriate to use the ratio inulin/chloride of 12.62/18.62 and apply it to the 13.02% chloride space. On this basis the extracellular space would be 8.82% and Table IV gives the calculated intracellular values. These are obtained from the

TABLE IV

Intracellular composition of muscle compared with plasma (calculated from data of Table II and an extracellular space of 8.82% muscle water.

	mg-ions per kg water							
	Na	K	Ca	Mg	Cl	SO_4	P*	Total
Muscle cells	17.9	130	2.01	13.8	12.7	1.06	100	277
Plasma	296	7.2	2.95	3.48	276	3.11	2.37	591
Ratio: $\frac{\text{muscle}}{\text{plasma}}$	0.060	18.1	0.68	4.0	0.046	0.34	42.2	0.47

* mg-atoms.

whole muscle analyses of Table II by subtracting values of plasma ions in 88.2 g of the total muscle water from the ionic concentrations of whole muscle, and recalculating the intracellular concentrations, now in 911.8 g water, to a kg water basis.

These intracellular concentrations show the normal pattern of high potassium and phosphate, and low sodium and chloride of muscle cells. Potassium and magnesium are the only cations to become concentrated in the cells, the former to eighteen times and the latter to four times the level in the plasma. Cellular sulphate and calcium remain low, calcium at about a seventh of the magnesium concentration. The ratios of K_i/K_o and Cl_o, Cl_i are respectively 18.1 and 21.7.

Muscle juice. The pale fawn juice centrifuged or pressed from muscle has an ionic composition similar to that of whole muscle when the analyses are given on a kg solvent water basis, the only marked differences being in their lower Ca (−43%) and Mg (−19.5%) values (Table V). Similarly the muscle juice and

TABLE V

Composition of muscle juice, centrifuged or pressed muscle and whole muscle [mg-ions (mM) per kg solvent water].

Ion	Whole muscle	Muscle juice	Centrifuged or pressed muscle	Difference		Difference	
				Whole muscle—muscle juice	*t*-test	Centrifuged muscle—muscle juice	*t*-test
Na	42.5 ± 9.1 (5)	40.1 ± 8.9 (5)	43.4 ± 9.3 (5)	2.40	3.27* (2.78)	3.36	3.24* (2.78)
K	128 ± 8.0 (6)	124 ± 7.5 (6)	132 ± 8.9 (6)	3.7	2.24 (2.57)	7.7	2.33 (2.57)
Ca	2.60 ± 0.56 (5)	1.49 ± 0.38 (5)	3.06 ± 0.64 (5)	1.11	5.25* (2.78)	1.58	5.31* (2.78)
Mg	11.81 ± 0.94 (5)	9.51 ± 0.76 (5)	13.41 ± 0.90 (5)	2.31	4.00* (2.78)	3.90	8.63* (2.78)
Cl	41.9 ± 7.6 (6)	44.9 ± 7.5 (6)	40.4 ± 7.9 (6)	−2.98	2.71* (2.57)	−4.43	3.72* (2.57)

* The mean difference between the paired observations is significant at $P = 0.05$, calculated t exceeding the value from table t given in brackets.

Mean water contents mg per kg are: original muscle 748 ± 6.4 (9), muscle juice 885 ± 2.4 (20), centrifuged muscle 699 ± 5.2 (20). ± = S.E. ( ) = N.

the piece of muscle from which it had been centrifuged or expressed differed only slightly except for marked reductions of Ca and Mg in the juice. The minor differences in the other ions are, however, significant at the 95% level, except those for K, and the Cl in the juice is higher in contrast to the lower values of the cations.

The purpose of these analyses was to demonstrate any ion binding which would be apparent by reductions in the ions of muscle juice compared with those in whole muscle and in centrifuged muscle. Extracellular fluid in muscle and muscle juice complicates any calculations of binding since it may form a different proportion of the solvent water in the two cases. In only one specimen was the inulin space determined in both muscle and muscle juice, the ratios found being 1.00 : 1.16. Using 10% and 11.6% extracellular volumes in the water of whole muscle and muscle juice, respectively, one can calculate the composition of muscle cells and that of the cellular component of the juice (Table VI). This calculation suggests that about half of the cellular Na and Ca and a fifth of the Mg are held back when muscle is centrifuged, perhaps bound in complexes by the structural proteins, actin and myosin, and possibly by the sarcolemma. None of the K would appear to be bound, and only 8% of the Cl. It is improbable that even this amount of Cl is

TABLE VI

Approximate amount of ion-binding (based on data of Table V), using extracellular volume of 10% and 11.6% in muscle and muscle juice, respectively.

	mg ions (mM) per kg water				
	Na	K	Ca	Mg	Cl
Muscle cell	14.3	141	2.56	12.7	15.9
Cellular portion of muscle juice	6.5	139	1.30	10.3	14.6
Bound ions (%)	55	1.4	49	19	8

bound. A slightly smaller extracellular volume in the muscle juice (11.2%) would result in negligible Cl binding (0.6%), while still leaving the same proportions of Ca and Mg bound, slightly less Na (45%) bound, and still only 1% of the K.

Organic constituents of plasma and muscle

Nitrogenous constituents. In Table VII are set out analyses of 7 nitrogenous constituents (excluding protein) in plasma and muscle: urea, trimethylamine oxide, betaine, free amino acids, creatine, creatinine and ammonium ions. On the average about 87.4% of the NPN of plasma, and 87.5% of the NPN of muscle are accounted for by these constituents, and if are added the ATP-N (see Table VIII) and amide-N (latter 3.27 and 5.72 mM in plasma and muscle respectively), about 92% of the NPN of muscle is attributable to known compounds and ions. While concentrations of urea in plasma and muscle are high but very similar, those of muscle TMAO are 3 times as high as plasma values. Because of these high values in muscle and the large amounts of the nitrogenous bases creatine and

TABLE VII

Nitrogenous constituents of plasma ana muscle.

	mM per kg water							
	Urea	TMAO	Betaine	Creatine	Creatinine	Amino-N	NH_4	Total NPN*
Plasma	308	72.4	9.1	0.126	0.046	11.6	0.40	838
S.D.	31.3	15.0	13.5	0.09	0.009	3.9	0.46	93.3
N	7	7	4	3	3	4	3	4
Muscle	333	180	100	68.2	1.02	108	4.7	1447
S.D.	18.8	31.6	33.5	8.3	0.81	24.0	3.8	89.2
N	7	7	7	13	3	7	4	7

* mg-atoms.
TMAO = trimethylamine oxide.
NPN = non-protein nitrogen.

betaine, together with the 10-times larger concentration of amino acids, muscle NPN is greater than plasma NPN by a factor of about 1.7.

Creatine is present in muscle partly as creatine phosphate, and this bound creatine was found in 3 estimations to be about a half of the total creatine (25.6, S.D. 6.2, compared with 62.9, S.D. 5.4 mM per kg water).

The phosphorus compounds. Phosphate compounds in ice-cold trichloroacetic acid extracts of muscle were separated into four fractions, inorganic phosphate, adenosine triphosphate (ATP), creatine phosphate and a fourth fraction, containing hexose phosphate *etc.*, by subtracting the sum of the first three from the total acid-soluble P. In the 3 specimens examined the labile creatine phosphate forms about 28% of the total P, inorganic phosphate 33%, ATP 11% and the remaining fraction 27% (Table VIII).

TABLE VIII

Acid-soluble phosphate fractions in muscle.

mg-ions per kg water				
Inorganic phosphate	Creatine phosphate	Adenosine triphosphate	Hexose phosphates, etc	Total phosphate
28.0 S.D. 5.9 (3)	23.9 6.2 (3)	9.6 0.79 (3)	22.9 5.5 (3)	84.4 5.9 (3)

Creatine phosphate as measured here from phosphate fractionation should be of the same order as the bound creatine determined by the α-naphthol diacetyl method. Values using the latter method for the same specimens showed broad agreement, 32, 26 and 19 mM per kg water as against 27, 28 and 17 mM as creatine phosphate.

Minor organic and inorganic constituents. Glucose and glycerol contribute little to the osmotic concentration of muscle. Glycerol values in muscle were below 1 mM [0.77, S.D. 0.22 (3)]. Of 5 specimens in which both the plasma and muscle glucose were determined, the values were within 4% of each other only in two cases. Means were 6.29, S.D. 3.76 (7) in plasma, and 5.06, S.D. 2.61 (8) in muscle. Total CO_2 in muscle was 7.08, S.D. 0.40 (5), the mean HCO_3 component being 6.81 at pH 7.5, and plasma inorganic P was 1.58, S.D. 0.88 (5).

Osmolality of plasma and muscle

From a summation of the analyzed ions and organic constituents we can see how far the totals of plasma and sea water, and of plasma and muscle, agree with each other (Table IX), and these totals can be compared with the measured osmolality of other specimens. The data are from three male specimens in which ions, nitrogenous compounds, glucose and glycerol were studied in both plasma and muscle. Neglecting for the moment the osmotic coefficients of ions and molecules, and the possibility that some of the ions are bound, it is seen that the plasma total of ions and molecules of 1116 is only 3% higher than the sea water ions. Inorganic and organic ions, urea and trimethylamine oxide form 89% of the total. Compared with plasma the total concentration of muscle is 7.7% higher. A reduc-

TABLE IX

Osmolality of plasma, whole muscle and sea water (mean of 3♂ specimens).

Constituent	mg-ions + mM per kg water			Osmotic coefficient	milliosmoles per kg water		
	Plasma	Muscle	Sea water		Plasma	Muscle	Sea water
Inorganic ions, phosphate *etc.*	606	291	1083	0.90	545	262	975
Urea	314	332	—	0.96	301	319	—
Trimethylamine oxide	76	172	—	1.19	90	205	—
Betaine	—	101	—	1.115	—	113	—
Amino acids and amides	13	118	—	?1.0	13	118	—
Creatine and Creatinine	0.2	61	—	?1.0	0.2	61	—
NPN unaccounted for	101*	121*	—	?1.0	?101	?121	—
Glucose and Glycerol	6	6	—	1.0	6	6	—
Total	1116	1202	1083		1056	1205	975

Creatine phosphate (23.6 mg-ions) subtracted from the ionic phosphate values of muscle since it is included in the total creatine.

* mg-atoms.

tion of ions to half those of the plasma is counterbalanced by more than a doubling of TMAO and a large increase in free amino acids and creatine, as well as the presence of 101 mM betaine.

It is possible that the unknown NPN of plasma and muscle may include compounds with more than one nitrogen atom per molecule, which would reduce both total concentrations. The dipeptides anserine and carnosine found in teleost muscle seem to be absent in dogfish muscle filtrates (unpublished work of C. B. Cowey and J. D. Robertson).

To convert the data to milliosmoles requires the use of osmotic coefficients which may depart from unity in both salts (ions) and organic compounds. These are given in the fifth column of Table IX. It is assumed that plasma and muscle ions have approximately the same osmotic coefficient as sea water ions. For a chlorinity of 18.00‰ (18.61 g Cl per kg solvent water), this can be calculated from the osmotic equivalence data of Robinson (1954) and the g-ionic concentration of sea water [1.1368 at Cl 19.00‰ as given by Sverdrup, Johnson and Fleming (1942)]. The value at Cl 18.00‰ is 969.9/1074.0 = 0.903, this being the osmotic equivalence in milliosmoles divided by the total mg-ions (mM) by analysis. The urea coefficient is from Robinson (1954), betaine from Smith and Smith (1940) and trimethylamine oxide by own determination as no value was found in the literature. Like the other dipolar compound betaine the value obtained for trimethylamine oxide exceeded unity substantially, being 1.19. It was determined on a molal solution of trimethylamine oxide dihydrate (Eastman chemical) by the vapour pressure method given in the introduction, using for comparison an organic standard of molal DL-alanine, which is an almost ideal solute with an osmotic coefficient of 1.003 (Robinson, 1952). Correction was made for the fact that an assay of the TMAO solution showed the compound to be 99.0% pure, as determined from its N (micro-Kjeldahl) and trimethylamine components (99.3% and 98.7%, respectively, of the theoretical).

A value of 1.0 has been taken for the average osmotic coefficient of the amino acids. At 1 molal the coefficient is 0.928 for glycine (Smith and Smith, 1937) and 1.046 for proline (Smith and Smith, 1940), but apart from alanine just mentioned, no published values for the amino acids have been found. In *Squalus* muscle the most abundant of the free amino acids are proline, glycine, taurine, alanine and sarcosine (C. B. Cowey and J. D. Robertson, unpublished). Proline, glycine and alanine formed on the average 68% of the total amino acids (104 mM per kg water) in 3 specimens, and using the coefficients 70.3 millimoles becomes 70.6 milliosmoles.

After conversion of all the data to milliosmoles the total concentration of plasma comes to 1056, 8.3% higher than the sea water in which the specimens were living (*cf.* Table I, where six male specimens of *Squalus* were hyperosmotic by 2.8%). The muscle concentration at 1205 milliosmoles is 14.1% higher than that of the plasma. Regarding the unknown non-protein nitrogen of the plasma a small fraction may be betaine (Table VII). Some of the remainder may be in peptide form, or even be present as a trace of non-precipitated protein; either or both of these possibilities would reduce the NPN unaccounted for and so also the calculated osmolality of the plasma. To get a calculated osmolality of plasma similar to that found by direct vapour pressure measurement of other specimens would require some 54 of the 101 mg-atoms N to be present in compounds of negligible osmotic concentration, that is, of high molecular weight.

The same suggestion may be put forward for the apparently excessive calculated osmotic concentration of muscle; some of the 121 mg-atoms NPN may be present in macromolecular form. A slight correction can, however, be made to it from knowledge of the free amino acids. The calculation of unaccounted for NPN was made on the assumption that the 112 mg-atoms α-amino N atoms found in these 3 specimens were from amino acids with a single N. In fact, from unpublished work the presence of lysine (2 N-atoms), histidine (3) and arginine (4) means that the 112 mM amino acids contain 121 mg-atoms N. The total of 121 mg-atoms NPN unaccounted for then becomes 112, and total milliosmoles for the muscle 1196.

The last factor to be considered is binding of ions. In plasma this is probably negligible, concerning a fraction of the 2.95 mg-ions calcium. In muscle binding it concerns inorganic ions and possibly some of the organic phosphate ions. On a basis of the binding of 45% Na, 1.4% K, 49% Ca, 19% Mg and 0.6% Cl of the intracellular ions (Table VI and text), a muscle value of 291 mg-ions (Table IX, based on Tables II and VIII) and an extracellular space of 8.82% of muscle water, the concentration of these ions in whole muscle, 212 mg-ions, is reduced by 12 mg-ions or 11 milliosmoles. Thus quantitatively the binding of these ions in *Squalus* muscle is not very important. The total ions are now (262–11) = 251 milliosmoles, bringing the total osmotic concentration from 1196 (corrected from 1205) to 1185. This total is still 12% higher than that calculated for the muscle, which may be compared with the 3.1% difference found between muscle juice and plasma (Table I).

As a general check on the validity of converting the analytical data to milliosmoles, using osmotic coefficients, a solution closely resembling *Squalus* plasma was made up, containing salts, urea and TMAO. Its composition in mM per kg water was NaCl 296, KCl 7.2, $CaCl_2$ 2.9, $MgCl_2$ 3.5, Na_2SO_4 5.7, $NaHCO_3$ 6.8, urea 289 and TMAO 71. The total molality of the salts came to 656.3 which, with an

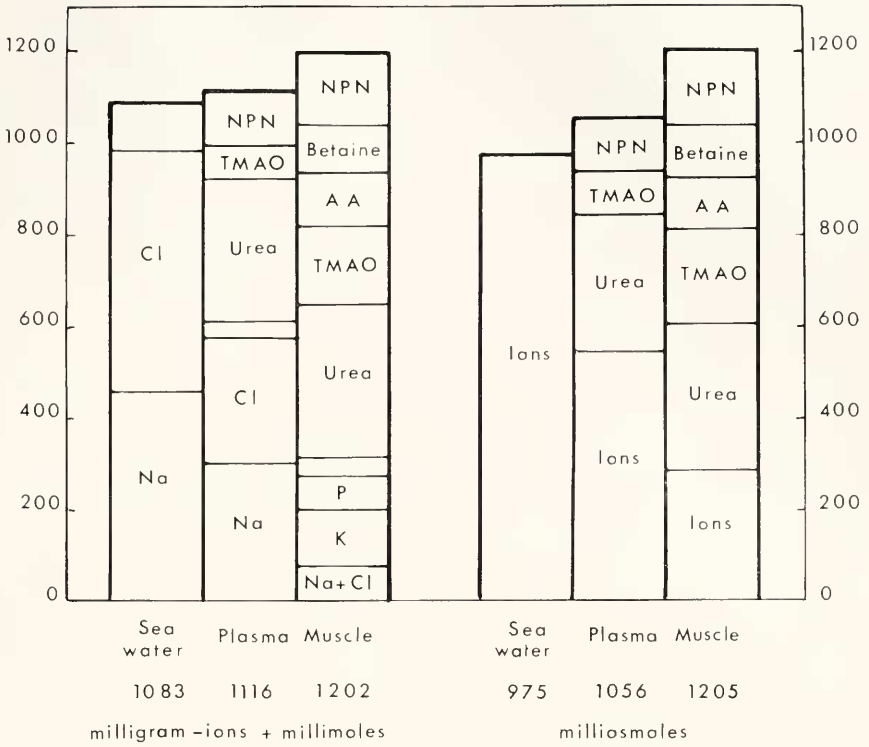

FIGURE 1. Composition of plasma and muscle of *Squalus acanthias* in relation to sea water; AA—free amino acids; TMAO—trimethylamine oxide; NPN—remaining non-protein nitrogen (in mg-atoms). The unlabeled sections in the columns are the other ions of plasma and muscle. Scale on left—ions and molecules as analyzed, scale on right—milliosmoles as calculated using osmotic coefficients.

osmotic coefficient of 0.903, is equivalent to 592.6 milliosmoles. Urea of 289 mM gives 277.4 milliosmoles (coefficient of 0.96) and TMAO of 71 mM equals 84.5 milliosmoles (coefficient of 1.19). Thus the estimated molality is 954.9 milliosmoles for an artificial plasma (minus protein) containing 1016.3 millimoles of salts and nitrogenous compounds. The osmolality, using the Krogh-Baldes vapor pressure thermoelectric method and NaCl standards, was found to be 948 milliosmoles, a difference of only 0.7%.

Figure 1 shows the principal constituents of plasma and muscle. The balance of NPN unaccounted for has been left uncorrected and is in mg-atoms. The chief features of the muscle compared to plasma are (1) the great reduction in muscle ions, the increase in K being far outweighed by decreases in Na and Cl, and (2) the great increase in TMAO, amino acids and betaine.

DISCUSSION

It seems well established that the blood serum or plasma of elasmobranchs is slightly hyperosmotic to sea water (Holmes and Donaldson, 1969). In the spiny

dogfish the writer's values, about 3% higher than the medium, are rather lower than most of the previous data for this species. Certainly the concentration of blood plasma in the *Squalus* from the Clyde sea-area shows a smaller mean difference from that for specimens of the same species from Salisbury Cove, Maine, where Burger (1967) gives a further value slightly below that of Burger and Hess (1960) of 1000 milliosmoles compared with 930 in sea water (+ 7.5%).

Comparison of the major ions in Table II with fairly complete analyses of the blood plasma or serum of *Squalus* from the eastern coasts of the United States and Canada shows good agreement. The values of Rodnan, Robin and Andrus (1962) and those of Macallum (1910), the latter obtained by classical gravimetric methods, in brackets, are Na 263 (296), K 4.1 (7.4), Ca 3.3 (4.3), Mg 1.55 (6.4), and Cl 249 (295). Macallum's data have been recalculated to mg-ions per kg water, those of Rodnan *et al.* (1962) left as mg-ions per liter. A 4% increase to get the data of Rodnan *et al.* in terms of weight of solvent water would bring the analyses even nearer to each other. The chief differences are in K and Mg.

Potassium values in plasma or serum are particularly prone to be higher than normal if the slightest tinge of hemoglobin indicates some slight hemolysis and leakage of K from the red blood cells. Cserr and Rall (1967) claim that the lowest values in the spiny dogfish are obtained if sampling from the caudal vein is immediate. Normal values of K 4.1 ± 0.12 mg-ions per liter rise 50% if the sampling is delayed 7–15 minutes after the fish is taken from the water. Despite complete lack of obvious hemolysis, the mean value of K 7.2 in Table II may be rather high. A further 2 ♂♂ gave 5.8 and 6.4, while 3 ♀♀ gave a mean of 4.8 (4.11, 4.38, 5.84), these values being in mg-ions (m. equiv.) per liter.

Lactate is an ion which shows considerable variation. The values of 6.0 and 8.3 mg-ions in 2 ♂♂ (mean 7.2 of Table II) and the lower figure of 2.9 in a ♀ may be compared with data given by Murdaugh and Robin (1967), in which elevated values in the laboratory specimens (sex unstated of 7.9 ± 5 (18) mg-ions per liter contrasted with lower values of 4.5 (10) in those kept in live cars in the dock area, and still lower, 0.94 ± 0.3 (11) in freshly captured specimens sampled within a minute.

Total inorganic ions of plasma in *Squalus* come to only 52% of the osmolarity in the analyses of Rodnan *et al.* (1962) and to 54% of the osmolality (based on concentrations per kg solvent water) in the present work (Table IX). Another value based on freezing points of original serum and ashed serum made up to its original volume is 53% (Macallum, 1910). Nitrogenous compounds, particularly urea, trimethylamine oxide, and to a minor extent free amino acids and perhaps betaine, are responsible for most of the balance, since glucose is relatively insignificant.

Urea values in *Squalus*, 288–350, mean 308 ± 31.3 (± = S.D.) mM per kg water (Table VII) may be compared with 357 ± 32 mM per liter based on an unstated number of plasma analyses by Rodnan *et al.* (1962), and 4 values, range 347–352 mM per liter of Burger and Hess (1960). Trimethylamine oxide at 72.4 ± 15, range 54–95 mM per kg water (Table VII) is in complete agreement with the data of Cohen, Krupp and Chidsey III (1958), 71 ± 10 (N = 39) mM per liter. A single value of 64 mM has been given for the serum of the North Pacific form of *Squalus acanthias* ('*S. suckleyi*') by Norris and Benoit (1945).

The only comprehensive data on dogfish plasma with which the present work on *Squalus* may be compared are those of Doolittle, Thomas and Stone (1960) on the smooth dogfish *Mustelus canis*. In a total of 1037 mM per kg water, ions account for 56%, urea 33% and TMAO 9.4%. The two nitrogenous compounds are rather higher than in *Squalus*, mean urea being 342 mM and TMAO 97 mM, but the individual ions are very similar. If the osmotic coefficients in Table IX are applied to the data, ions come to 527, urea to 328 and TMAO to 115 milliosmoles which together with glucose add up to 983. This may be compared with their directly estimated 962 and 910 milliosmoles (from freezing-point depression) for *Mustelus* plasma and the sea water in the tank at Woods Hole. Agreement is good, but total NPN was not estimated.

A study on the electrolytes of muscle in *Squalus* has been made by Robin, Murdaugh and Weiss (1964) who analyzed 10 fish for Na, K and Cl. They calculated intracellular concentrations on the basis of exclusion of Cl from muscle cells, using the chloride space (muscle Cl as % plasma Cl, both on a water content basis) as a measure of extracellular space. The chloride spaces found were 14 ± 2% (range 11–17), which may be compared with those from the data of Tables II and III of 13.0 and 18.6% (means for 3 and 4 dogfish respectively). Plasma Na and K (means) were 240 and 3.6, with whole muscle means of 55.4 (38–75) and 162 (104–216) mg-ions per kg water. Thus while Na values are comparable with data in Tables II and V, most of the muscle K concentrations of Robin *et al.* (1964) exceed those in this paper, mean 125 (9), range 95–146. Calculated intracellular concentrations are higher for K, 187 ± 42, compared to 130 and 141 in the present work, and higher also for Na, 29.5 ± 9, as against 17.9 and 14.3 (Tables IV and VI). If Cl is present inside muscle cells, as is probable from a comparison of chloride with inulin space (Table III), the calculated intracellular concentrations in the American specimens would be altered, resulting in a lower K and a higher Na. However, some variation in ionic concentrations must be expected and the two sets of data are broadly consistent. Some variation in extracellular and intracellular ions must arise from a degree of flexibility in the proportion of ions and nitrogeneous molecules making up the osmotic concentration. An exceptionally low urea and NPN in the plasma of one specimen was offset by a higher Cl, indicating some such mutual adjustment.

Juice expressed from muscle has an osmotic concentration averaging 3% higher than that of plasma (Table I). It would seem that this reflects a slight breakdown of labile constituents during the pressing or centrifugation of the muscle, so that *in vivo* muscle is probably almost isosmotic with the plasma.

While the concentrations of K and of both inorganic and organic phosphate are high in muscle, the Na and Cl are so low that the proportion of the osmotic concentration due to ions is only about a half what it is in plasma. Nitrogenous compounds make up the balance. Urea is only silghtly higher than in plasma, in the specimens analyzed 333 and 308 mM per kg water, but there are large increases in creatine, mean 68.2 mM from less than 1 mM in the plasma, TMAO 180 mM as against 72 mM, betaine 100 mM from very small amounts, and free amino acids 108 mM compared to 12 mM (Table VII).

Data comparing blood and muscle concentrations of these compounds in *Squalus* seem to exist only for TMAO. Goldstein, Hartman and Forster (1967) give

3 comparisons in which muscle concentrations (per kg water) were 3 times those of the plasma, 225, 154 and 216 mM compared to 76, 45 and 78 mM, respectively. These values are all within the ranges given in Table VII.

Extensive data on the nitrogenous extractives of *Squalus acanthias* muscle have been given by Vyncke (1970). Recalculation of his mean data and ranges for nitrogenous compounds on a millimolal basis gives urea 415 (351–480), TMAO 189 (165–212), betaine 11.0 (3.3–31.7), creatine 51.1 (26.5–65.7), creatinine 6.2 (2.0–10), ammonium 18.4 (5.6–33.7), α-amino N 61 $\pm$ 13 in mature, 74.2 $\pm$ 14 in immature dogfish. The analyses in Table VII are in general agreement with these means and ranges, except that amino acids and betaine are much higher, and urea, creatinine and ammonium ions are in the lower ranges. In the present work creatinine and ammonium ions were determined immediately, since their values rise in extracts kept in the refrigerator. In trichloroacetic acid solution creatine slowly changes to a mixture of creatinine and creatine, the values of creatinine rising as creatine falls.

The question of betaine must be considered. In muscle of three elasmobranchs including *Squalus*, Shewan (1953) found that TMAO and betaine were the two largest of the nitrogenous extractives, but no absolute values for betaine were given. He later (Shewan, 1961) gave a figure of 1500 mg per kg muscle. Assuming a water content of 75%, this amounts to only 17 mM per kg water. Vyncke's (1970) maximum value is only 32 mM. Much time was spent on getting a fairly satisfactory method of analysis and the writer considers his value of 100 (52–145) mM to be valid. This amount is comparable to that found in decapod crustacean and cephalopod muscles (*e.g.*, 99 mM in *Sepia* whole muscle, Robertson, 1965). *Squalus* plasma seems to have a low betaine concentration, < 4 mM. An exceptional 29.2 mM was found in a dogfish with a muscle value of 121 mM but other plasma values were 0.4, 3.0 and 3.7 (mean 9.1, Table VII).

The principal aim of this work was to get a comprehensive analysis of muscle and plasma and to see how far the totals of the principal osmotic constituents agreed with each other and with direct measurements of plasma and muscle osmotic concentration. On the basis of chemical estimations, the calculated osmolality of known ions and compounds (means of 3 ♂♂ dogfish) is 955 in plasma compared with 975 milliosmoles in sea water. But a further 101 mg-atoms of plasma NPN has to be added, which, if present in compounds with 1 N atom per molecule, would give a calculated value 8.3% higher than the sea water. Possibly some of the nitrogen is in peptide form, which would reduce the difference. The actual mean difference in 6 ♂♂ was + 3% (Table I).

For muscle the calculated osmolality of known ions and molecules is 1084 milliosmoles, but it has a further 121 mg-atoms of N unaccounted for. Some of this is probably peptide-N, as Vyncke (1970 finds 59 $\pm$ 13 mg-atoms in *Squalus* muscle. Disregarding it for the moment, the known ions and compounds (1084) come to a value 2.7% higher than that of the plasma, in good, perhaps fortuitous, agreement with the measured + 3% in other specimens (Table I). Including it, the total is 1205, + 8.3% on the plasma value. Presumably this higher value would be reduced (1) if any breakdown of labile compounds from the *in vivo* condition could be allowed for, (2) if some of the N is in petpide form, and (3) if a proportion of any of the ions and molecules is bound in complexes with the cellular proteins. Cohen *et al.* (1958) have shown by dialysis that no binding of TMAO

occurs in the plasma of *Squalus*. It is possible that some binding of nitrogenous constituents does occur in muscle, such as part or all of the ATP. Although it is inferred that some of the cellular Na, Ca and Mg is bound (Table VI), the concentrations of these ions are so small that the reduction due to binding (minus 11 milliosmoles) causes only a minor alteration to the osmolality of whole muscle.

A brief comparison may be made between dogfish and members of two other groups, *Myxine glutinosa*, a marine cyclostome isosmotic with sea water, and *Latimeria chalumnae*, the marine crossopterygian. According to Griffith, Unminger, Grant, Pang and Pickford (1974) ions in the blood serum of *Latimeria* come to 435.4 mg-ions per liter, and with urea 377 mM, TMAO 122 mM, amino acids 15.6 mM and glucose 6.6 mM, the total is 957. Using the osmotic coefficients in Table IX this total equals 922 milliosmoles, in complete agreement with the directly measured osmolarity of 932 milliosmoles per liter, sea water being 1035. No excess of NPN was found, the N of the known compounds exceeding the total NPN (856 mg-atoms) by 4%. The nitrogenous compounds of *Latimeria* muscle from a frozen specimen included 422 mM urea and 290 mM TMAO (Lutz and Robertson, 1971). Thus these constituents in both blood and muscle are higher in *Latimeria* than in *Squalus*. Contrasting with both of these fishes is the cyclostome *Myxine* in which nitrogenous constituents are practically absent from the plasma, ions forming about 99% of the osmotic concentration (Robertson, 1966). *Myxine* muscle is also different from that of these fishes, in that urea is practically absent (1.5 mM). It does contain TMAO 87 mM and betaine 65 mM per kg water, concentrations which are much lower than in *Squalus*. It makes up for this by having a high concentration of free amino acids, 291 mM, so that the total nitrogenous consituents form about 55% of the osmotic concentration in *Myxine*, compared to about 69% in *Squalus*.

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Summary

Comprehensive analyses of the osmotic consituents of plasma and muscle of *Squalus acanthias* have been made. In plasma mean concentrations of ions and molecules (mg-ions or mM per kg water) were Na 296, K 7.2, Ca 2.95, Mg 3.48, NH_4 0.4, Cl 276, SO_4 3.11, HCO_3 6.8, lactate 7.2, urea 308, trimethylamine oxide (TMAO) 72.4, amino-N 11.6, total NPN 838 mg-atoms. In muscle mean concentrations were Na 42.4, K 119, Ca 2.09, Mg 12.9 Cl 35.9, SO_4 1.24, lactate 23.8, total P (mg-atoms) 91.3, urea 333, TMAO 180, betaine 100, creatine 68.2 (26% bound as creatine phosphate), amino-N 108, NH_4 4.7, total NPN 1447 mg-atoms. In other specimens fractionation of the acid-soluble compounds in muscle gave inorganic P 28.0, creatine phosphate 23.9, ATP 9.6, remaining phosphate 22.9 (total 84.4 mg-ions).

Using osmotic coefficients calculated osmotic concentration of muscle exceeded that of plasma, and possible reasons for this are discussed. Directly determined

osmotic concentrations were sea water 965, plasma (6 ♂♂) 993, and muscle juice 1023 milliosmoles.

Some estimates of intracellular ionic concentrations were obtained from measurements of inulin space in muscle. Analysis of muscle juice enabled approximate estimates of ion-binding. About half the Na and Ca and nearly one fifth of the Mg in muscle cells appears to be bound, but little of the Cl and K.

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